Virology 276, 148–160 (2000)
doi:10.1006/viro.2000.0544, available online at http://www.idealibrary.com on IDEAL®

Suppressor Mutations That Allow Sindbis Virus RNA Polymerase to Function with Nonaromatic Amino Acids at the N-Terminus: Evidence for Interaction between nsP1 and nsP4 in Minus-Strand RNA Synthesis

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Received April 26, 2000; returned to author for revision May 25, 2000; accepted July 20, 2000

The alphavirus RNA polymerase, nsP4, invariably has a Tyr residue at the N-terminus. Previously we reported that the N-terminal Tyr residue of nsP4 of Sindbis virus, the type species of the genus *Alphavirus,* can be substituted with Phe, Trp, or His without altering the wild-type phenotype in cultured cells but that other substitutions tested, except for Met, were lethal or quasilethal. Here we report the identification of two suppressor mutations in nsP4 (Glu-191 to Leu and Glu-315 to Gly, Val, or Lys) and one in nsP1 (Thr-349 to Lys) that allow nsP4 with nonaromatic amino acids at the N-terminus to function at 30°C. The suppressor mutation at nsP4 Glu-315 occurred most frequently. All three suppressor mutations suppressed the effects of Ala, Arg, or Leu at the N-terminus of nsP4 with almost equal efficiency and thus the effect of the suppressing mutation is independent of the nsP4 N-terminal residue. Reconstructed mutants containing nsP1-T349K or nsP4-E315G combined with Ala-nsP4 had a defect in minus-strand RNA synthesis at 40°C. A double mutant containing nsP4-Q191L combined with Ala-nsP4 was unstable and could not be tested for RNA synthesis because it reverted to temperature-independence too rapidly. Combinations of nsP1-T349K or nsP4-E315G with Leu, Arg, His, or any aromatic amino acid at the N-terminus of nsP4 also made the mutant viruses temperature sensitive. The results from this study and from a previous report on the shutoff of minus-strand RNA synthesis at 40°C with the nsP1-A348T mutation in *ts*11 suggests that the N-terminus nsP4 interacts with nsP1 during initiation of minus-strand RNA synthesis.

INTRODUCTION

Sindbis virus (SIN) is the prototype virus of the genus *Alphavirus,* a group of 26 arthropod-borne viruses, many of which cause disease in humans (Strauss *et al.,* 1995). In nature, SIN alternately infects mosquito vectors and higher vertebrates, primarily birds, although the virus does infect mammals, including humans. Some strains of SIN cause disease in humans characterized by fever, rash, and polyarthritis (Strauss and Strauss, 1994; Shirako *et al.,* 1991).

Following infection of a cell, the genomic plus-strand RNA is a messenger for the synthesis of two overlapping polyproteins, called P123 and P1234, that are processed by a virus-encoded protease within the polyproteins (reviewed in Strauss and Strauss, 1994). The first processing event in the full-length translation product, P1234, is an autoproteolytic cleavage *in cis* to release nsP4, which is the viral RNA polymerase. This cleavage is absolutely required for viral RNA replication (Shirako and Strauss, 1994). The proteins resulting from this cleavage, P123 and nsP4, form a minus-strand replicase. Subsequent cleavage of P123 *in trans* by the viral nonstructural protease first results in the conversion of the replicase to an intermediate form (nsP1 + P23 + nsP4) that is capable of making both plus-strands and minus-strands and then into the fully cleaved form (nsP1 + nsP2 + nsP3 + nsP4) that can only make plus-strands (Shirako and Strauss, 1994; Lemm *et al.,* 1994). Host proteins are also thought to be components of the replicases (Barton *et al.,* 1991; Kuhn *et al.,* 1991).

The N-terminus of nsP4 is Tyr in all alphaviruses examined (Strauss and Strauss, 1994; Rumenapf *et al.,* 1995; Lee *et al.,* 1997; Shirako and Yamaguchi, 2000), and we have shown that only Tyr, Trp, Phe, or His at the N-terminus allow wild-type or near wild-type function of nsP4: viruses with Tyr-nsP4, Trp-nsP4, Phe-nsP4, or His-nsP4 grow well at both 30 and 40°C and form large plaques (Shirako and Strauss, 1998). Met-nsP4 also results in viable virus, but the virus forms small plaques and is attenuated. Any other amino acid at the N-terminus renders the mutant nonviable and no plaques are formed following transfection of cells. However, upon prolonged incubation at 30°C after transfection of RNA transcripts from mutants carrying Ala, Thr, Leu, Arg, Gln, or Cys at the N-terminus of nsP4, revertants arose. Many of these revertants were same site revertants in which the mutant N-terminal residue had changed to Tyr, Trp,

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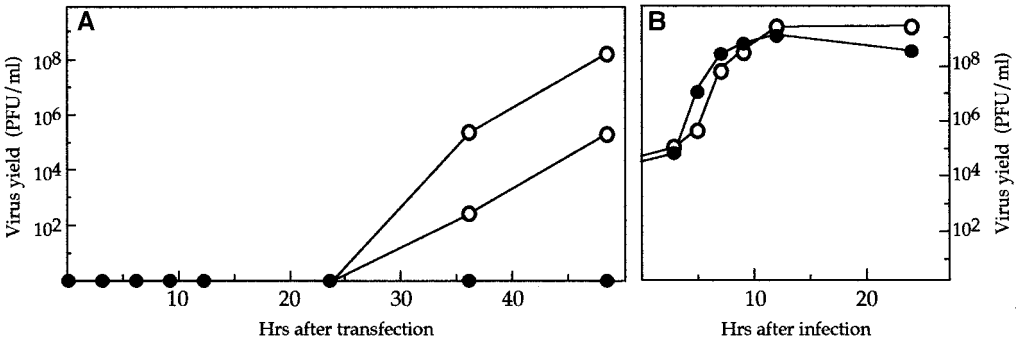

FIG. 1. Emergence of pseudorevertant viruses from cells transfected with mutant Ala-nsP4 RNA. In (A), virus titers from two transfection experiments are plotted. In (B), rescued pseudorevertant virus was used to infect cells and the average titers from duplicate experiments are shown. Open circles, incubation at 30°C; filled circles, incubation at 40°C.

His, or Phe if it was possible to convert the mutant codon into a codon encoding one of these amino acids by a single nucleotide substitution (Shirako and Strauss, 1998). Other rescued revertant viruses retained the original N-terminal mutations; these revertants formed smaller plaques and many were temperature sensitive, and we assumed that suppressor mutations must be present somewhere in the genome that allowed nsP4 to function with an otherwise nonpermitted residue at the N-terminus.

In this paper, we report the mapping and identification of three second-site suppressor mutations, one in nsP1 and two in nsP4, that allow viruses having nsP4 containing N-terminal Ala, Arg, or Leu to replicate and produce progeny virions, albeit in a temperature-sensitive manner. The nsP1 mutation and one of the nsP4 mutations are of particular interest because they occur immediately adjacent to (nsP1 Thr-349 → Lys) or at the same residue as (nsP4 Gln-191 → Leu) previously mapped temperature-sensitive mutations (Ala-348 → Thr, Wang *et al.,* 1991; Gln-191 → Lys, Sawicki *et al.,* 1990). Our results provide evidence that nsP1 in the polyprotein P123, and probably in the partially cleaved complex containing nsP1 + P23 as well, interacts with nsP4 for the initiation of minus-strand RNA synthesis.

RESULTS

Emergence of revertant viruses from the Ala-nsP4 mutant

We reported previously that transfection of transcripts of mutants having Ala at the N-terminus of nsP4 into chicken monolayer cells resulted in the emergence of revertant viruses after prolonged incubation at 30°C. These revertants were attenuated, many being temperature sensitive, and retained Ala at the N-terminus of nsP4 (Shirako and Strauss, 1998). The kinetics of emergence of revertant viruses are illustrated in Fig. 1A where two independent experiments are plotted. Infectious virus was not detected 24 h after transfection at either 30 or 40°C, but was detected 36 h after transfection at 30°C and the titer had increased at 48 h. At 40°C, no viable virus was detected even after 48 h of incubation. In contrast, transfection with wild-type transcripts results in the release of substantial quantities of infectious virus by 10 h at either 30 or 40°C (not shown).

A stock of revertant virus harvested after 4 days of incubation at 30°C, when all cells showed cytopathic effects, had a titer of about 10^9 PFU/ml at 30°C (where small plaques were formed) and about 10^8 PFU/ml at 40°C (where minute plaques were formed). This stock was used for the analysis of virus growth at 30 and 40°C (Fig. 1B), using a multiplicity of infection of about 50 for cells incubated at 30°C and about 5 for cells incubated at 40°C. At both temperatures virus grew well, reaching around 10^9 PFU/ml at 12 h postinfection (plaque titration was performed at 30°C). The fact that the revertant stock produced only 10% as many plaques at 40 as at 30°C indicates that the majority of the revertant virus population rescued after transfection of the Ala-nsP4 mutant at 30°C could grow only at 30°C. However, the fact that there was virus in this same stock that was able to infect cells and replicate well at both 40 and 30°C indicates that there was a second, minority population in the revertant virus stock that was temperature-independent.

Identification of second-site suppressor nsP1-T349K

To identify second-site suppressor mutations in the revertant virus genome, several different approaches were used. In one approach, a revertant virus stock produced after transfection of transcripts from pToto1101S.4A, which has a Ser codon in place of the opal termination codon near the end of the nsP3 gene as well as the Tyr → Ala mutation at the N-terminus of nsP4, was used. The Ala-nsP4 mutant is convenient for these studies because no same-site revertants have been found to arise, since two nucleotide changes are required to obtain Tyr or one of the other acceptable amino acids. The revertant virus stock, called AS30-1, was further propagated in chicken cells at 30°C and the char-

TABLE 1

Mapping of Suppressors of Mutations in the N-Terminus of Sindbis nsP4

Selection Revertant name[a]	Revertant phenotype: Titer pfu/ml (30/40°C)	Revertant phenotype: Plaque size[b] (30/40°C)	Constructs[c] examined	Location of suppressor: Fragment	Protein	aa change
Revertants selected by prolonged incubation at 30°C						
AS30-1	$10^9/10^8$	Sm/Mn				
1 of 9 constructs			B	B	nsP1	T349 → K
2 of 4 constructs			C	C	nsP1	T349 → K
4 of 4 constructs			D,E	No mutations found		
Revertants selected after passage at 40°C						
A40-1	$10^9/10^9$	Sm/Sm				
1 of 19 constructs			3′	*Spe*I–*Nsi*I	nsP4	Q191 → L
Multiple constructs			A,B,C,D,E,F,5′	No mutations found		
Revertants selected by limiting dilution at 30°C						
A30-1.1, A30-1.3	10^9/none	Sm/—	5′,3,D,E,F	*Nsi*I–*Sac*I	nsP4	E315 → G
L30 14.1	10^9/none	Sm/—	5′,3,D,E,F	*Nsi*I–*Sac*I	nsP4	E315 → G

[a] Mutants are named as follows: AS30-1 means the nsP4 N-terminus is Ala, the opal codon following nsP3 is replaced with Ser, incubation was at 30°C, and this is the 1st transfection experiment with this mutant construct. Variable numbers of constructs from each transfection were examined. In the case of limiting dilution, the additional number defines the clonally selected virus (0.1). For the 40°C series, a revertant stock obtained after transfection at 30°C was further passaged at 40°C in an attempt to obtain a temperature-independent revertant.

[b] Plaque size on chicken embryo fibroblast monolayers incubated at 30 and 40°C were scored as large (Lg), small (Sm), or minute (Mn). For the series AS30-1 and A40-1, the plaque phenotype is that of the mixed yield from the initial transfection or from the subsequently passaged virus; for revertants selected by limiting dilution, the phenotype is that of the cloned virus stock.

[c] Constructs used for mapping are shown in Fig. 2B.

acteristics of the resulting stock are shown in Table 1. The virus in this stock was purified by sucrose density gradient centrifugation, viral RNA was extracted and used to make double strand cDNA, and various restriction fragments from this cDNA were used to replace the corresponding fragments in pToto1101.4A (Fig. 2). Transfection of transcripts from one of nine constructs containing the *Hin*dIII–*Hin*dIII cDNA fragment from the rescued mutant (construct B in Fig. 2B) and two of four constructs containing the *Nde*I–*Bgl*II cDNA fragment (construct C) resulted in formation of small plaques at 30°C but none at 40°C, presumably mapping the suppressing mutation to the overlap region of these two fragments. Transcripts from constructs D and E were nonviable at either temperature (four constructs tested in each case). The 5′ terminal region (Fig. 2b, construct A (and the 3′ terminal region (Fig. 2B, construct F) were not examined in this case.

The entire inserts of the three viable constructs were sequenced and each showed a single nucleotide substitution at position 1105, from C to A, causing a change at nsP1 position 349 from Thr (ACG) to Lys (AAG) (Fig. 2C, constructs a and b). Thus, a single mutation in nsP1, T349K, was found to suppress the Ala-nsP4 mutation at 30°C but not at 40°C. Note that the effect of the suppressor is independent of whether Ser or opal is present at the end of nsP3; the original revertant was found in the variant containing Ser but the mapping was performed in the opal variant. The mapping results are summarized in Table 1.

Identification of nsP4-Q191L as a suppressor

A revertant virus stock prepared by transfection of pToto1101.4A transcripts at 30°C (this Ala-nsP4 construct has the opal codon near the end of nsP3) was passaged twice at 40°C in an attempt to identify a suppressor that will function at 40°C. The passaged virus formed small plaques at both 30 and 40°C with similar titers, around 10^9 PFU/ml, indicating that the virus population became fairly uniform after the passages at 40°C (Table 1, stock A40-1). The virus was then propagated at 30°C and purified, and extracted RNA was used for the preparation of double-strand cDNA. Restriction fragments representing the entire genome (except for 24 nucleotides at the 5′ terminus that were derived from the primer oligonucleotide used for PCR amplification) (regions A to F in Fig. 2B) were tested for the presence of suppressor mutations. For each region, more than three independent clones were constructed, and transcripts were tested for plaque formation directly after transfection. However, none of the clones produced virus capable of forming a plaque at either 30 or 40°C. We considered the possibility that two or more second-site mutations were required to suppress the Ala-nsP4 mutation in a temperature-independent manner. To test this, restriction fragments were combined to produce clones 5′ and 3′ (Fig. 2B) as well as full-length clones. However, none of the clones produced transcripts that gave rise to viruses able to plaque at 40°C. Thus the suppressing mutations that allow

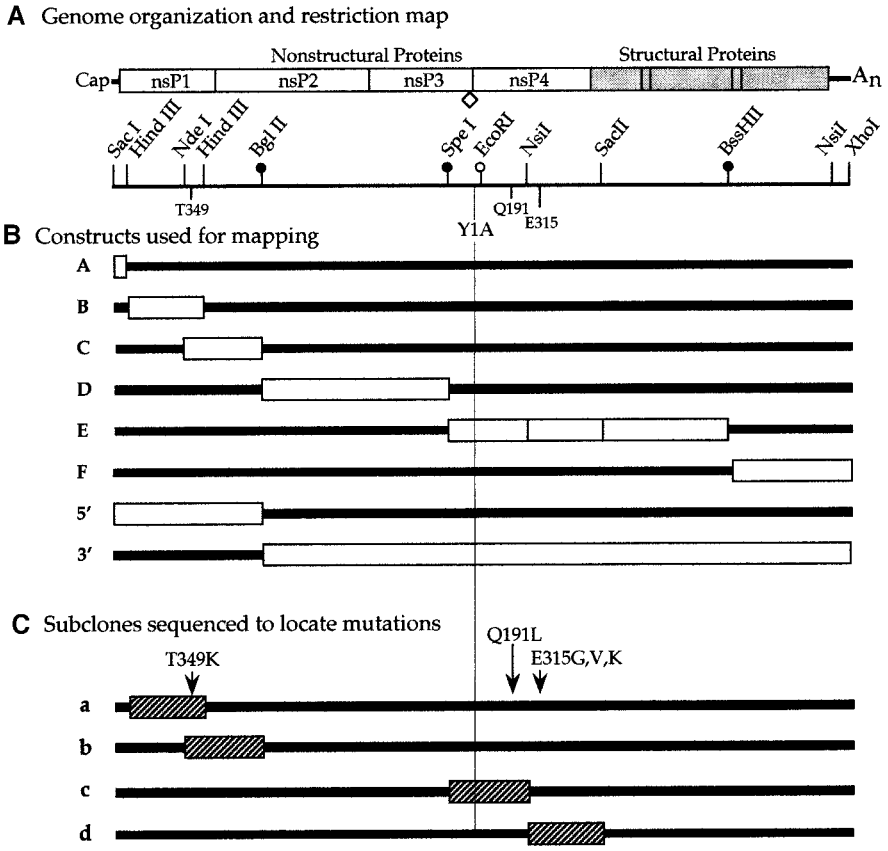

FIG. 2. Mapping of suppressor mutations in pseudorevertant virus genomes. (A) The genome organization of Sindbis virus RNA is illustrated. The positions of restriction enzyme recognition sites used in the mapping or in various constructions are indicated below. The *Sac*I site near the 5′ end is present immediately upstream of the SP6 promoter used to transcribe RNA *in vitro,* and the *Xho*I site near the 3′ end is immediately downstream of the poly(A). The *Bgl*II, *Spe*I, and *Xho*I sites, indicated with solid stalked symbols, are unique in the viral genome; the *Eco*RI site, open symbol, was used in some cloning steps. The open diamond marks the position of the opal codon just upstream of the N-terminus of nsP4. Also shown are the three positions at which suppressor mutations were found. (B) Constructs used for mapping. Rectangular boxes indicate cDNA derived from revertant virus RNA and solid lines are derived from the pToto1101.4A vector. The vertical line marks the position of Ala at the N-terminus of nsP4 (Y1A). Vertical lines within the box in construct E indicate the internal restriction sites used for fine-structure mapping. (C) Subclones sequenced to locate mutations. Hatched boxes were fully sequenced after the mapping analysis. The suppressor mutations that were found are shown with labeled arrows above the constructs.

growth and plaque formation at 40°C cannot lie within the coding region of the genome.

While examining the clones 5′ and 3′ (Fig. 2B), however, we did identify a suppressor that functioned at 30 but not at 40°C. Twelve independent clones of 5′ and 9 independent clones of 3′ were tested for plaque formation directly after RNA transfection. None produced plaques at 40° but 1 of the 3′ clones produced plaques at 30°C. The causative mutation was mapped between the *Spe*I site at position 5262 and the *Nsi*I site at 6457. Sequence analysis revealed a single nucleotide difference at position 6340 from A to T, causing a change at residue 191 of nsP4 from Gln (CAG) to Leu (CTG) (Fig. 2C, construct c). These mapping results are summarized in Table 1.

Why the nsP4-Q191L suppressor, which is able to suppress the Ala-nsP4 mutation only at 30°C, was present in the virus stock is not entirely clear. The most likely scenario is that it arose during the original passage at 30°C, allowing the virus to grow at this temperature, and was carried along during passage at 40°C, during which a second suppressor was selected that allowed growth at 40°C. Separation from the second suppressor during mapping would then lead to the observed result. As shown below, virus containing the nsP4-Q191L mutation is unstable and reverts readily to a temperature-insensitive variant, consistent with this hypothesis. Also consistent with this hypothesis is that whereas the other suppressors identified by us during mapping render the virus temperature sensitive when combined with wild-type residues at the N terminus of nsP4, virus containing nsP4-Q191L is not temperature sensitive when combined with Tyr-nsP4, i.e., this mutation in itself does not render the virus temperature sensitive (see below).

Identification of suppressors at nsP4 residue 315

In another attempt to identify suppressor mutations, we isolated individual revertants by end-point dilution of virus stocks rescued after transfection of the Ala-nsP4

TABLE 2

Location of Suppressors of Mutations in the N-terminus of Sindbis nsP4 by Sequence Analysis of Cloned RT-PCR Fragments

Selection Revertant name[a]	Revertant phenotype		Amino acid at[c]	
	Titer pfu/ml (30/40°C)	Plaque size[b] (30/40°C)	nsP1 T349	nsP4 E315
A30-12	$10^9/10^7$	Sm/Sm	wt (9/9 clones)	E315 → G (2/7 clones) E315 → K (2/7 clones) wt (3/7 clones)
A30-13	$10^9/10^7$	Sm/Sm	wt (9/9 clones)	E315 → G (7/7 clones)
A30-14	$10^9/10^8$	Sm/Mn	wt (8/8 clones)	E315 → V (8/8 clones)
A30-15	$10^9/10^6$	Sm/Sm	wt (9/9 clones)	E315 → G (4/9 clones) E315 → K (4/9 clones) wt (1/9 clones)
AS30-12	$10^9/10^8$	Sm/Sm	wt (9/9 clones)	E315 → V (5/9 clones) wt (4/9 clones)
A30-1	$10^9/10^8$	Sm/Mn	wt (8/8 clones)	See Table 1
A30-11	$10^9/10^8$	Sm/Sm	wt (8/8 clones)	wt (7/7 clones)
AS30-11	$10^9/10^9$	Sm/Sm	wt (9/9 clones)	wt (9/9 clones)
AS30-13	10^9/none	Lg/—	wt (9/9 clones)	wt (7/7 clones)
AS30-14	$10^9/10^9$	Sm/Mn	wt (9/9 clones)	wt (7/7 clones)
AS30-15	$10^9/10^8$	Sm/Sm	wt (9/9 clones)	wt (9/9 clones)

[a] Mutants were named as in Table 1. All mutant populations in this table were obtained by prolonged incubation at 30°C.

[b] Plaque phenotype is of the mixed yield after the 30°C incubation and defined as in Table 1.

[c] Short fragments containing possible suppressor mutations were amplified from the RNA of the mixed virus population by RT-PCR, using two sets of primers, one for the nsP1 region and one for the nsP4 domain. These fragments were cloned into plasmids. Multiple clones from each series were sequenced. Using this method, no suppressors could be identified from any of the transfection yields shown below the dashed line.

mutant RNA at 30°C. Since the titers of these virus stocks were 10 times higher at 30 than at 40°C, we expected that revertant virus clones which are viable only at 30°C should be isolated more frequently than those which are also viable at 40°C. Two virus clones were isolated that formed small plaques at 30°C but no plaques at 40°C. These clones were propagated at 30°C and the extracted RNA from purified virus preparation was used for mapping as before (Figs. 2B and 2C). None of the cDNA clones possessing the 5′-terminal 2.3 kb (clone 5′) formed plaques at either 30 or 40°C, but all five cDNA clones containing the 3′-terminal 9.4 kb (clone 3′) from the two revertant viruses formed small plaques at 30°C (but no plaques at 40°C). The 9.4-kb 3′ insert was further mapped into shorter fragments and the causative mutation was found to lie between the *Nsi*I site at position 6457 and the *Sac*II site at position 7713 (Fig. 2C, construct d). The 1.3-kb *Nsi*I–*Sac*II fragment was sequenced, and only a single nucleotide difference from the wild type was identified, an A to G change at position 6712 that results in an amino acid change in nsP4 at position 315 from Glu (GAA) to Gly (GGA). The results are summarized in Table 1.

We also isolated revertant virus clones from stocks derived after transfection of transcripts from the Leu-nsP4 and Arg-nsP4 mutants. One revertant from Leu-nsP4 was mapped and we found the same nsP4-E315G mutation as those from Ala-nsP4 clones (Table 1). Thus this suppressor is able to suppress both Ala-nsP4 and Leu-nsP4.

Frequency of occurrence of the three different suppressors

We identified three suppressor mutations, nsP1-T349, nsP4-Q191, and nsP4-E315, by mapping of the revertant genomes described above. To investigate the frequency of occurrence of these three suppressor mutations, we sequenced short domains surrounding one or more of these three residues from a number of independently isolated revertant virus stocks. Only 30°C stocks were examined, because these three suppressor mutations are able to suppress Ala-nsP4 only at 30°C. Most of the revertant stocks examined were uncloned stocks produced by extended incubation of transfected cells at 30°C. Table 2 shows results from six independent revertant stocks that were rescued after transfection of transcripts from pToto1101.4A (the Ala mutant that contains the opal codon at the end of nsP3) and five additional independent revertant stocks rescued after transfection with pToto1101S.4A (the Ala mutant with the serine codon). In a few cases, stocks were cloned by limiting dilution at 30°C and cloned revertants were thus examined. These cloned revertants were derived from mutants Leu-nsP4 (three revertant viruses) or Arg-nsP4 (two revertant viruses), and the results are shown in Table 3. In either case, virus RNA was isolated from revertant stocks

TABLE 3

Location of Suppressors of Mutations in the N-Terminus of Sindbis nsP4 by Direct Sequencing of RT-PCR Fragments

Selection / Revertant name[a]	Revertant phenotype		Amino acid at[c]		
	Titer pfu/ml (30/40°C)	Plaque size[b] (30/40°C)	nsP1 T349	nsP4 Q191	nsP4 E315
Revertants selected by limiting dilution at 30°C					
Leucine at the N-terminus of nsP4					
L30-12.1	10^9/none	Sm/—	wt	wt	E315 → K
L30-12.2	10^9/none	Sm/—	wt	wt	wt
L30-14.2	10^9/none	Sm/—	wt	wt	E315 → G
Arginine at the N-terminus of nsP4					
R30-13.1, R30-13.2	10^9/none	Sm/—	wt	wt	E315 → G

[a] Mutants were named as in Table 1.

[b] Plaque phenotypes are of the pure culture of each clonally selected variant and defined as in Table 1.

[c] Short fragments containing possible suppressor mutations were amplified from the viral RNA by RT-PCR, using sets of primers specific for the nsP1 region of the nsP4 domain. The PCR amplified DNA was sequenced directly.

and PCR was used to amplify selected regions, using appropriate primers. The PCR DNA was either cloned into a plasmid and then sequenced (if the revertant stock was uncloned as in Table 2) or sequenced directly (if the revertant stock had been cloned as in Table 3). For ease of presentation, the results are described in the order nsP1-T349, nsP4-Q191, and nsP4-E315, that is, all results for nsP1-T349 are described before moving to nsP4-Q191, etc. This requires some moving back and forth among the tables but makes for a more streamlined and logical presentation than moving back and forth among the mutants.

The 11 uncloned stocks in Table 2 were examined for the amino acid at nsP1 position 349 by amplifying the 5′-terminal 2.3-kb region of the viral RNA and cloning the PCR product into vector pSCV12. The area around nucleotide 1105, the nucleotide change in nsP1-T349K, was sequenced. From the 11 stocks a total of 96 clones were examined and all had the wild-type sequence at this position (Table 2). Similarly, the 3 cloned revertants from Leu-nsP4 and the 2 from Arg-nsP4 had the wild-type sequence at this position (Table 3). Thus the nsP1-T349K suppressor is present only rarely. It was found in only a single revertant stock (of 15 independent revertant stocks examined for this change), and even in this case it formed only a minority of the revertant population (Table 1).

We also examined the Leu-nsP4 and Arg-nsP4 revertants for the presence of nsP4-Leu191. All five revertants examined had the wild-type residue at this position (Table 3). Thus this suppressor also appears to be uncommon in the revertant population. Of six stocks examined for this mutation by mapping (which examines the entire nonstructural protein coding region) or by sequencing around this position, this suppressor was found in only a single clone from one population of revertants (Table 1). As discussed above, this mutant may have been found in this revertant population because of the method used to produce it.

In contrast to the results for nsP1-T349 and nsP4-Q191, however, examination of revertant stocks for the residue at nsP4 position 315 showed that changes at Glu-315 occurred commonly. Changes at this position were found in at least some clones from 12 of 18 independently passaged mutants that were examined, and almost one-half of the individual suppressed variants examined had changes at this residue (Tables 1–3). Furthermore, although changes of Glu (GAA) to Gly (GGA) were found repeatedly, changes to Lys (AAA) and Val (GTA) were also found (Tables 2 and 3). In fact, two of the stocks examined had a mixture of Gly and Lys at this position (as well as the wild-type Glu), showing that both arise frequently and appear to be about equally effective in suppression (since one has not outcompeted the other during the experiment). Since changes at nsP4-E315 were also found in variants arising from Leu-nsP4 and Arg-nsP4 as well as Ala-nsP4 (Tables 1–3), it is clear that changes at this position can suppress not only Ala-nsP4 but also Leu-nsP4 and Arg-nsP4.

Of minor historical interest is the fact that although changes at nsP4-E315 are found most commonly, it was the last of the three suppressors that was identified in this study. The fact that it was not found in the 40°C stocks examined is to be expected in light of our subsequent finding that it is temperature sensitive (see below). More interesting is the fact that it was not present in stock AS30-1 (Table 1). This suppressor seems to be uncommon in stocks of revertants from mutants having the serine codon, having been found in only one of six independent revertant stocks from such mutants that were examined at this position. In contrast, it was found in eight of nine stocks of revertants examined that arose from mutants having the opal stop codon.

It is noteworthy that in half of the cases no suppressor was identified and it seems certain that other suppressors must exist. No suppressor active at 40°C was found, although stocks that grow at this temperature

TABLE 4

Change in Plaque Phenotype during Passage of Sindbis Pseudorevertants Containing Ala-nsP4

	Primary RNA transfection[a] Phenotype[b]			Secondary virus infection[d] Phenotype[b]		
Mutations	Temp. (°C)	Titer (PFU/ml) (30/40°C)	Plaque size (30/40°C)	Temp. (°C)	Titer (PFU/ml) (30/40°C)	Plaque size (30/40°C)
nsP1 T349K	30	10^9/—[c]	Sm/—	30	$10^9/10^3$	Sm/Mn
	40	—/—	—/—	40	$10^3/10^3$	Mn/Mn
nsP4 E315G	30	10^9/—[c]	Sm/—	30	$10^9/10^3$	Sm/Mn
	40	—/—	—/—	40	$10^3/10^3$	Mn/Mn
nsP4 Q191L	30	$10^9/10^7$	Lg/Sm			
	40	—/—	—/—			

[a] After primary RNA transfection, cells were incubated at 30 or 40°C for 48 h and the medium was harvested for plaque assay.

[b] Lg, large plaque; Sm, small plaque; Mn, minute plaque; —, no plaques at any dilution.

[c] No plaques at any dilution, but cytopathic effect was seen when monolayers were infected with medium diluted 10-fold.

[d] Cells were infected with virus from the primary transfection at an m.o.i. of 10 based on the 30°C titer and incubated at 30 or 40°C for 24 h, and the medium was harvested for plaque assay.

arose. In addition, suppressors were not found in many of the variants from the 30°C stocks, indicating that additional suppressors active at 30°C must exist.

Stability of Ala-nsP4 mutants containing suppressing mutations

cDNA constructs containing Ala-nsP4 in combination with one of the three suppressing mutations were used to prepare virus for the purpose of characterizing the suppressed mutants in a uniform background. We first examined the stability of the mutants (Table 4). Cells transfected with transcripts of Ala-nsP4 suppressed by nsP1-T349K developed cytopathic effects within two days of incubation at 30°C but cells incubated at 40°C did not, confirming that this revertant virus is not viable at 40°C. Virus recovered from the 30°C incubation formed small plaques at 30°C and had a titer of 10^9, but no plaques were formed at 40°C. However, infection of chicken cells at 30 or at 40°C with virus from the 30°C stock resulted in cytopathic effects at both temperatures. Progeny virus from the second 30°C passage had a titer of 10^9 PFU/ml in a plaque assay at 30°C, forming small plaques, whereas at 40°C it had a titer of 10^3 and formed minute plaques. Progeny virus from the 40°C incubation, on the other hand, had a titer of 10^3 when plaqued at either 30 or 40°C, forming small plaques at 30°C and minute plaques at 40°C. Similar results were obtained when Ala-nsP4 was suppressed by nsP4-E315G. Thus these two suppressed mutants were not stable and acquired additional mutation(s) upon passage at 30°C. However, virus capable of growing at 40°C represents $<10^{-8}$ of the yield after the first passage and this first passage virus is thus suitable as an infecting stock for the additional experiments described below.

After transfection of cells with transcripts of Ala-nsP4 suppressed by nsP4-Q191L, cells incubated at 30°C developed cytopathic effects whereas those incubated at 40°C did not, confirming that this suppressed mutant was not viable at 40°C. However, the progeny virus from the 30°C passage formed large plaques at 30°C (titer 10^9) and formed small plaques at 40°C (titer 10^7). Therefore, the original double mutant, which is not viable at 40°C, gained additional unidentified mutation(s) quickly after 30°C incubation and additional experiments to further characterize this suppressor were not feasible.

Ala-nsP4 mutants have a defect in minus-strand RNA synthesis at 40°C

Viral minus-strand RNA synthesis in chicken cells infected with Ala-nsP4 mutants suppressed by either nsP1-T349K or nsP4-E315G was analyzed by RT-PCR as described previously (Shirako and Strauss, 1994). In this assay, a 0.9-kb RT-PCR product is produced from minus-strand RNA. Different numbers of PCR cycles (10, 15, 20, 25) were used so as to quantitate approximately the relative amounts of minus-strand RNA under the different conditions. The results are shown in Fig. 3.

The level of accumulation of minus-strand RNA at 30°C was very similar for wild-type virus and for the Ala-nsP4 mutants suppressed by either nsP1-T349K or nsP4-E315G. Minus-strand RNA was detectable by 1 h in the 25-cycle reaction in all three cases (Fig. 3). By 3 h a faint band was visible even in the 15-cycle reaction and was easily detectable in the 20-cycle reaction. By 9 h a fairly strong band was present in the 15-cycle reaction. No further accumulation was seen in the 24-h sample from the mutants, indicating that minus-strand RNA synthesis has been shut down (Sawicki *et al.,* 1981). Thus the suppressed mutants are able to synthesize minus-strand RNA with about the same efficiency as wild-type virus at this temperature (Fig. 3).

At 40°C a different picture emerges. In wild-type in-

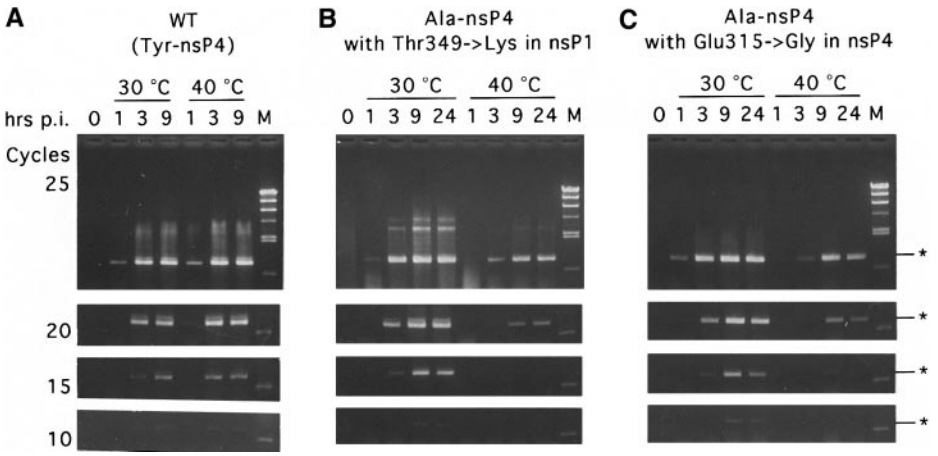

FIG. 3. RT-PCR analysis of minus-strand RNA accumulation in chicken cells infected with (A) the wild-type virus, (B) the nsP1-T349K/Ala-nsP4 mutant, or (C) the nsP4-E315G/Ala-nsP4 mutant. Cells were inoculated with virus at a multiplicity of 10 at 4°C for 1 h in T25 flasks. the inoculum was then removed and prewarmed medium was added. Cells were harvested immediately (lanes marked 0) or after 1, 3, 9, or 24 h of incubation at 30 or 40°C, as indicated by the temperature and time shown above each lane. Intracellular RNA was extracted and RT-PCR was performed. Minus-strand RNA gives rise in this assay to a 0.9-kb fragment derived from the 3′ end of full-length minus-strand RNA (indicated by the asterisks on the right). Lane M is λ DNA digested with *Hin*dIII. Different numbers of PCR cycles were used in an effort to quantitate approximately the amount of minus-strand RNA present, and the numbers in the left margin of A indicate the numbers of cycles during PCR amplification. The entire gel is shown for the 25-cycle PCR reaction, but only the 0.9-kb region is shown for the 10-, 15-, or 20-cycle reactions.

fected cells, minus-strand RNA was detectable by 1 h in the 25-cycle reaction. The amount of minus-strand RNA was higher at 3 h, as is easily seen in the 15- and 20-cycle reactions, but then did not change much over the next 6 h (compare the 15-cycle reactions at 3 and 9 h). This result agrees with previous findings that minus-strand RNA is shut off at about 3 h at 40°C (Sawicki *et al.,* 1981) (shutoff occurs somewhat later at 30°C). However, synthesis of minus-strand RNA at 40°C in cells transfected with either the nsP1-T349K or the nsP4-E315G double mutant was delayed and less RNA accumulated (Fig. 3). No minus-strand RNA was detectable at 1 h. At 3 h a faint band was present in the 25-cycle reaction. More RNA was present at 9 h, as seen most readily in the 20-cycle reaction. Wild-type levels of minus-strand RNA never accumulated, as seen most strikingly in the 15-cycle reactions. Thus both suppressed Ala-nsP4 mutants have defects in minus-strand RNA synthesis at 40°C. It seems likely that the delayed production of smaller amounts of minus-strand RNA at 40°C by the suppressed mutants results from a limited ability of these mutants to make minus-strand RNA at this temperature, but synthesis resulting from the emergence of variants able to replicate at 40°C, as described above, cannot be completely ruled out.

The suppressor mutations are general suppressors that render the virus ts

The nsP1-T349K, nsP4-Q191L, and nsP4-E315G mutations were combined with Tyr, Phe, Trp, His, Met, Ala, Leu, or Arg codons at the 5′-terminus of the nsP4 gene, and transcripts from the various constructs were transfected into chicken monolayer cells and directly assayed for the ability to form plaques at 30 and 40°C. The results are shown in Table 5. The first conclusion to be drawn from these data is that all three suppressor mutations suppress not only Ala-nsP4 at 30°C but also Leu-nsP4 and Arg-nsP4 (see also Table 1, which has additional data for Arg-nsP4 and Leu-nsP4). All three of these N-terminal mutations are otherwise lethal (Shirako and Strauss, 1998), and thus, where tested, the effect of suppression is independent of the amino acid at the N-terminus of nsP4. The second conclusion is that all three suppressors also give rise to viable virus at 30°C when combined with Tyr, Phe, Trp, and His at the N-terminus of nsP4, which are permitted residues at this position. The suppressors also give viable virus when combined with Met, which is semi-permitted (virus with Met-nsP4 in an otherwise wild-type background is viable but the phenotype is not wild type). The third conclusion is that nsP1-T349K and nsP4-E315G render the virus ts and unable to replicate at 40°C. Even when combined with the wild-type Tyr-nsP4, the mutant is ts and these two mutations are thus ts mutations in and of themselves. The nsP4-Q191L mutation, in contrast, is not ts when combined with wild-type or near wild-type residues at the N-terminus of nsP4 (Tyr, Trp, His; Phe was not tested), but the suppressed double mutants with Ala-, Leu-, Arg-, or Met-nsP4 are ts.

Although nsP1-T349K combined with Tyr-nsP4 is ts, cells transfected and maintained at 40°C in liquid medium do develop cytopathic effects. Thus, although this virus is unable to form plaques at this temperature, cells die upon prolonged incubation, perhaps because the

TABLE 5

Plaque Phenotypes[a] of Sindbis Variants with Different nsP4 N-Termini Combined with Various Suppressor Mutations[b]

nsP4 N-terminus	Suppressor mutations			
	nsP1 T349K (30/40°C)	nsP4 Q191L (30/40°C)	nsP4 E315G (30/40°C)	ts11 nsP1 A348T (30/40°C)
Permitted termini				
Tyr (wt)	Lg/—	Lg/Lg	Lg/—	Lg/—
Phe	Lg/—	nd	Lg/—	—/—
Trp	Lg/—	Lg/Sm	Lg/—	Lg/—
His	Lg/—	Lg/Sm	Lg/—	Lg/—
Semi-permitted terminus				
Met	Lg/—	Sm/—	Lg/—	—/—
Nonpermitted termini				
Ala	Sm/—	Sm/—	Sm/—	—/—
Leu	Sm/—	Sm/—	Sm/—	—/—
Arg	Lg/—	Sm/—	nd	—/—

[a] Lg, large plaques; Sm, small plaques; —, no plaques; nd, not determined.

[b] Chicken cell monolayers were transfected with RNA from the different constructs, overlaid with solid media, and examined directly for the formation of plaques at 30 or 40°C.

virus replicates well enough that temperature-independent variants quickly arise. For other combinations, no cytopathology developed at 40°C under the conditions used here.

Studies with mutant ts11 (nsP1-A348T)

Since the nsP1-T349K mutation was positioned immediately adjacent to the previously identified mutation nsP1-A348T in ts11 (Hahn *et al.,* 1989), which has been shown to have a defect in minus-strand RNA synthesis (Wang *et al.,* 1991), we also examined the ts11 mutation. The nsP1-A348T mutation was combined with the various nsP4 N-terminal residues and the ability of the mutant viruses to form plaques at 30°C and 40°C was tested (Table 5). As previously described, this mutation renders the virus ts when combined with Tyr-nsP4 (the wild-type residue). Interestingly, a ts virus is also obtained with Trp-nsP4 and His-nsP4 but combination with Phe-nsP4, an otherwise permitted residue, is lethal, as is combination with the semi-permissive Met-nsP4. As shown in Table 5, the ts11 mutation does not suppress the effects of Ala, Leu, or Arg at the N-terminus of nsP4, and no plaques are formed at either 30 or 40°C.

DISCUSSION

Suppressor mutations in nsP1 and nsP4

We have identified two mutations in nsP4 and one in nsP1 that suppress the lethal effects of a nonaromatic amino acid at the N-terminus of nsP4. These mutations are illustrated schematically in Fig. 4. These three mutations appear to suppress the lethality of almost any nonpermitted amino acid at this position. Although we tested only N-terminal Ala, Arg, and Leu, these three amino acids are very different in terms of their properties, the first being small and neutral, the second large and basic, and the third bulky and hydrophobic, and suppression thus probably works for any amino acid at the N-terminus. In all three cases, the suppressed mutant is temperature sensitive, growing at 30 but not at 40°C.

The fact that the suppressors work with about equal efficiency with any of the three tested nonpermitted amino acids at the N-terminus of nsP4 is interesting in light of the fact that Leu and Arg are destabilizing amino acids for degradation by the N-end rule pathway (Varshavsky, 1992), whereas Ala is a stabilizing amino acid. This provides further evidence that the requirement for an aromatic amino acid or His at the N-terminus of nsP4 is not due to the instability conferred on nsP4 (de Groot *et al.,* 1991) but rather to a requirement for the ring structure. The ring structure could be required for the appropriate conformation of nsP4, for an interaction with other viral or cellular proteins, or because it is involved in the recognition of *cis*-acting elements in the viral RNA.

The three suppressors also give rise to viable virus when combined with the wild-type Tyr and with other aromatic residues but in two cases (nsP1-T349K and nsP4-E315G) the suppressor renders the virus temperature sensitive, as are the suppressed mutants. This finding is consistent with the finding that the suppressors apparently function with any amino acid at the N-terminus and suggests that the effect of the suppressor is to eliminate the necessity for some interaction that occurs between this N-terminal residue and some other component of the replication machinery that is normally required for RNA replication. We have previously shown

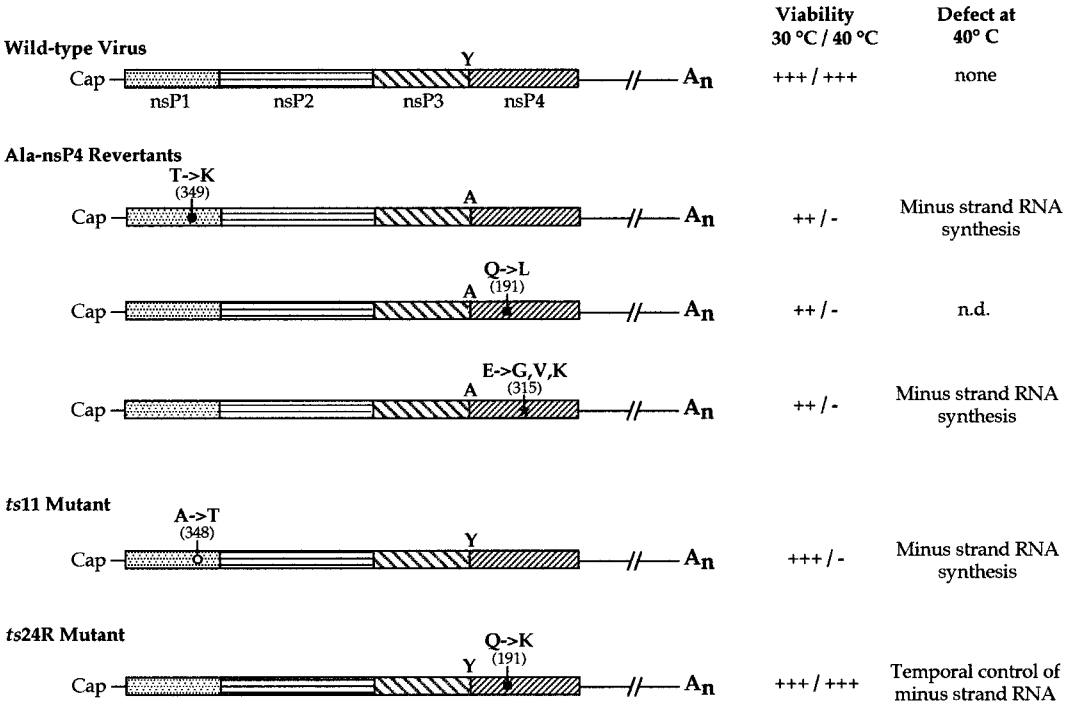

FIG. 4. Schematic representation of the location of the three suppressor mutations identified here and of the causative mutations of two previously characterized temperature-sensitive mutants in Sindbis virus nonstructural proteins. The phenotypes of the different mutants are shown at the right.

that cleavage of the precursor polyprotein to release the N-terminus of nsP4 is absolutely required for minus-strand RNA synthesis and probably for plus-strand RNA synthesis as well (Shirako and Strauss, 1994), which is also consistent with these observations.

Ala-nsP4 can function as an active RNA polymerase

nsP4 bearing N-terminal alanine is clearly functional in RNA replication. The nsP1 suppressor T349K allows Ala-nsP4 to function in RNA replication at 30°C leading to near wild-type production of virus. Furthermore, under Results we describe pseudorevertants that will form a plaque at 40°C but which still bear N-terminal Ala and whose nsP4 contains no suppressor mutation. Thus Ala-nsP4 is active at both 30 and 40°C as a core RNA polymerase. The lethality of N-terminal alanine in the otherwise wild-type virus therefore cannot result from the loss of the enzymatic activity of the nsP4 polymerase per se, but must be due to an inefficient interaction with other viral or cellular proteins or with *cis*-acting elements in the viral RNA, consistent with the logic of the preceeding paragraph.

Interaction of nsP1 and nsP4 for minus-strand RNA synthesis

The Ala-nsP4 mutant suppressed by nsP1-T349K makes minus-strand RNA at wild-type rates at 30°C but has a defect in minus-strand RNA synthesis at 40°C. When combined with wild-type Tyr-nsP4, nsP1-T349K alone renders the virus temperature sensitive, presumably because of the same defect in minus-strand RNA synthesis. It is of considerable interest, therefore, that the causative mutation in ts11 is the change nsP1-A348T, found immediately adjacent to the T349K mutation (Fig. 4). ts11 has been shown to have a defect in minus-strand RNA synthesis at 40°C (Sawicki *et al.,* 1981; Wang *et al.,* 1991). These data suggest that the nsP1 domain encompassing A_{348}–T_{349} interacts with the N-terminus of nsP4 and this interaction is required for the recognition of the promoter for minus-strand synthesis. Two forms of minus-strand replicase occur (Shirako and Strauss, 1994; Lemm *et al.,* 1994). The earliest form to appear contains the uncleaved polyprotein P123 + nsP4 and is produced by autocatalytic cleavage of P1234. This replicase is capable of efficient synthesis of only minus-strand RNA. The second form is produced by cleavage of the early replicase *in trans* at the bond between nsP1 and P23. Because of this requirement for cleavage *in trans,* production of this replicase, which can make both plus- and minus-strand RNAs, is delayed. We have previously found that the activity of the early replicase can be demonstrated readily by the PCR reaction used to produce the data in Fig. 3 (Shirako and Strauss, 1994). Therefore, the finding that production of minus-strand RNA is delayed at 40°C in the case of suppressed mutants (Fig. 3) must mean that the proposed interaction between nsP1 and nsP4 occurs in the early replicase, between nsP1 in P123 and nsP4. The fact that the accu-

mulation of minus-strand RNA never reaches wild-type levels makes it seem likely that an interaction between nsP1 and nsP4 continues to be required for minus-strand RNA synthesis in the partially cleaved replicase containing nsP1 + P23 + nsP4.

The two suppressor mutations in nsP4 are also implicated in synthesis of minus-strand RNA. We found that nsP4-E315G rendered the virus temperature sensitive for the synthesis of minus-strand RNA, having properties similar to the nsP1-T349K mutation. We were unable to test the defect in the suppressor nsP4-Q191L directly, but another mutation at nsP4 GIn-191 has been shown previously to be involved in minus-strand RNA synthesis. Sawicki *et al.* (1990) showed that the mutant nsP4-Q191K continued to synthesize minus-strand RNA at 40°C, whereas wild-type virus shuts down synthesis of minus-strand RNA at 3–4 h after infection. This mutant thus continues to recognize the promoter for minus-strand RNA after cleavage of P123 to form a plus-strand replicase (reviewed in Strauss and Strauss, 1994), which implies that the nsP4 residue Q191 is important for recognition of the minus-strand promoter.

Thus the three suppressor mutations identified in this study have been implicated, directly or indirectly, in recognition of the promoter for minus-strand RNA synthesis. We have no direct evidence dealing with the question of whether these mutations affect plus-strand RNA synthesis. However, previous work with ts11 and Q191K, which lie close to two of the suppressor mutations and affect minus-strand RNA synthesis, as described above, has produced no evidence that either affects plus-strand RNA synthesis. In fact, ts11 has been shown to be able to make plus-strand RNA at 40°C from previously formed minus-strand templates (Sawicki *et al.,* 1981). Similarly, the nsP4 mutant Q191K appears to make plus strands normally at 40°C. Thus there is no reason a priori to believe that the suppressor mutants identified by us also have a role in plus-strand synthesis. Although further study will be required to determine whether the N-terminal tyrosine of nsP4 does in fact play a role in plus-strand synthesis as well as minus-strand synthesis, we suggest that a primary function of the N-terminal tyrosine released by cleavage of the precursor polyprotein P1234 is in the recognition of the promoter for minus-strand synthesis, which requires its interaction with the domain in nsP1 containing residues A_{348}–T_{349}.

MATERIALS AND METHODS

Cells and medium

Secondary chicken embryo fibroblast cells in Eagle's minimum essential medium containing 3% fetal bovine serum were used for RNA transfection, virus propagation, and plaque assay throughout this study.

Sindbis viruses mutant in the N-terminus of nsP4

pToto1101.4A, 4L, 4R, 4M, 4W, and 4H, which have an Ala (GCC), Leu (CTC), Arg (AGG), Met (ATG), Phe (TTC), Trp (TGG), and His (CAC) codon, respectively, at the 5′-terminus of the nsP4 gene in place of the wild-type Tyr (TAC) codon have been described (Shirako and Strauss, 1998). The background of these clones is the pToto1101 full-length "infectious" cDNA clone (Rice *et al.,* 1987). pToto1101S.4A has a Ser codon (TCC) in the place of an opal termination codon (UGA) near the end of nsP3 (Li and Rice, 1989) in addition to the Tyr → Ala mutation at the N-terminus of nsP4.

Recovery and isolation of revertant viruses

In vitro transcripts from pToto DNA linearized with *Xho*I were transfected into chicken embryo fibroblast cells using DEAE–dextran as described previously (Shirako and Strauss, 1994). Transfected cells were incubated at 30°C for an appropriate period and the medium was harvested. If necessary, rescued virus was further passaged in chicken cells incubated at 30 or at 40°C. Virus clones were obtained by a limiting-dilution end-point method using a 96-well plate.

Mapping of suppressing mutations in pseudorevertants

Stocks of bulk virus or of virus clones were propagated at 30°C in chicken cells for 48 h. Virus was purified by differential centrifugation and sucrose density gradient centrifugation. RNA was extracted from the purified virus suspension and cDNA was synthesized using a primer, 5′ CCGCTCGAG$(T)_{42}$*GA* 3′ where the underlined sequence is an *Xho*I site and the two italicized nucleotides are complementary to the 3′-terminal nucleotides adjacent to the 3′-terminal poly(A) tract of Sindbis RNA, or an internal primer complementary to nts 2378–2397. Second-strand DNA was synthesized by the method of Gubler and Hoffman (1983). The mapping strategy was shown in Fig. 2B. The *Bgl*II–*Spe*I fragment (3.0 kb, region D), *Spe*I–*Bss*II fragment (4.5 kb, region E), *Bss*HII–*Xho*I fragment (region F), and *Bgl*II–*Xho*I fragment (region 3′) were directly cloned into pToto1101.4A vectors digested with the two boundary restriction enzymes. The *Hin*dIII–*Hin*dIII fragment (1.2 kb, region B) and *Nde*I–*Bgl*II fragment (1.2 kb, region C) were cloned into pToto1101.4A using an intermediate vector pSCV12 which contains the 5′ terminal *Sac*I–*Bgl*II fragment from pToto1101 (Shirako and Strauss, 1990). The 5′-terminal *Sac*I–*Hin*dIII fragment (region A) or *Sac*I–*Bgl*II fragment (region 3′) were prepared by RT-PCR using the internal minus-sense primer used for cDNA synthesis with GAGGAGCTC**ATTTAGGTGACACTATA***GATTGACGGCGTAGTACACACTATT* as a plus-sense primer, in which the underlined nucleotides form a *Sac*I site, the boldface nucleotides form an

SP6 promoter sequence, and the italicized nucleotides form the 5′-terminal 24 nucleotides of pToto1101-derived wild-type Sindbis virus genomic RNA. The *Sac*I–*Nde*I fragment was introduced into pToto1101.4A through the intermediate vector pSCV12, whereas the *Sac*I–*Bgl*I fragment was cloned directly into pToto1101.4A using the appropriate restriction enzymes. For fine mapping of region E, the *Spe*I–*Bss*HII fragment from Toto1101.4A was cloned into an intermediate vector pSCV34CE2 (Shirako and Strauss, 1998) and the *Spe*I–*Nsi*I, *Nsi*I–*Sac*I, and *Sac*II–*Bss*HII fragments were replaced with the corresponding fragments obtained from cDNA made to revertant virus RNA. The reconstituted *Spe*I–*Bss*HII fragment was cloned into *Spe*I–*Bss*HII-cut pToto1101.4A. The resulting pToto cDNA clones were linearized with *Xho*I and transcribed using SP6 RNA polymerase. Transcripts were transfected into chicken monolayer cells, which were overlaid with agarose and incubated at 30 or 40°C for 48 h before being stained with Neutral Red. When plaques were formed, the nucleotide sequence of the entire cDNA derived from the revertant virus was determined in order to identify suppressor mutations.

RT-PCR assay of minus-strand RNA

Minus-strand RNA in infected cells was determined as described previously (Shirako and Strauss, 1994). Briefly, chicken monolayer cells were infected at 4°C with virus at a multiplicity of 10. After removal of inoculum, prewarmed medium was added and the infected cells were incubated at 30 or 40°C for 1, 3, 9, or 24 h. Cells were harvested by trypsinization, pelleted by centrifugation, resuspended in phosphate-buffered saline, and lysed with 1% NP-40. Nuclei were removed by centrifugation and intracellular RNA was extracted by an SDS–phenol method, precipitated with ethanol, resuspended, and treated with DNase I. The RNA was again extracted and cDNA was synthesized using a plus-sense primer which had the sequence of nucleotides 1–20 of the genomic RNA. The cDNA was amplified by PCR using a minus-sense primer complementary to nucleotides 865–882 of the genomic RNA and the plus-sense primer above. After 10, 15, 20, and 25 cycles of PCR, a fraction of the reaction mix was taken and electrophoresed in a 0.8% agarose gel and stained with ethidium bromide. Extracts from cells that were infected at 4°C as above but not subsequently incubated were used as a control.

Construction of double mutants

The nsP1-T349K/nsP4-Y1A clone was digested with *Sac*I and *Bgl*II and the resulting 2.3-kb fragment was cloned into *Sac*I/*Bgl*II-digested pToto1101 or derivatives having Phe, Trp, His, Met, Leu, or Arg codons at the 5′-terminus of the nsP4 gene (Shirako and Strauss, 1998) to produce constructs having nsP1-T349K combined with various nsP4 N-terminal residues. The nsP4-Q191L mutation was combined with the wild-type Tyr or with Trp, His, Met, Leu, and Arg at the N-terminus of nsP4 by cloning the 0.6-kb *Eco*RI–*Nsi*I fragment from the nsP4-Y1A/Q191L clone (which contains the Q191L mutation) into an intermediate vector containing the 4.5-kb *Spe*I–*Bss*HII fragment (which contains the N-terminus of nsP4) from pToto1101 clones containing Tyr, Trp, His, Met, Leu, or Arg codons at the 5′-terminus of the nsP4 gene, followed by cloning the *Spe*I–*Bss*HII fragment into the *Spe*I/*Bss*HII-digested pToto1101 vector. The nsP4-E315G mutation was introduced into pToto1101.A by exchanging the *Nsi*I–*Sac*II fragment. It was then combined with Tyr-, Phe-, Trp-, His-, Met-, and Leu-nsP4 mutations as described for the nsP4-Q191L mutants.

ACKNOWLEDGMENT

This work was supported by Grant AI 10793 from the NIH.

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